Delayed expression of cytokines after reperfused myocardial infarction: possible trigger for cardiac dysfunction and ventricular remodeling

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Moro C, Jouan MG, Rakotovao A, Toufektsian MC, Ormezzano O, Nagy N, Tosaki A, Leiris J, Boucher F. Delayed expression of cytokines after reperfused myocardial infarction: possible trigger for cardiac dysfunction and ventricular remodeling. Am J Physiol Heart Circ Physiol 293: H3014–H3019, 2007. First published September 14, 2007; doi:10.1152/ajpheart.00797.2007.—Previous studies have shown that ischemia-reperfusion increases the production and release of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, and accumulated evidence indicates that cytokines are important mediators of infarct healing and cardiac remodeling. Indeed, in a rat model of myocardial infarction, TNF-α mRNA levels, measured in the noninfarcted region of the heart at 8 or 20 wk after infarction, were significantly higher than in sham-operated animals and correlated with left ventricular (LV) end-diastolic diameter (LVEDD), suggesting a possible involvement of TNF-α during the remodeling process of the heart (21). In addition, pathophysiologically relevant concentrations of TNF-α have been shown to induce LV dysfunction and dilation (3), therefore mimicking some aspects of heart failure. From these observations, the hypothesis that TNF-α and/or other cytokine overexpression might contribute to the progressive development of chronic heart failure after infarction has arisen. Nevertheless, decreasing the bioavailability of TNF-α in patients with heart failure has paradoxically not been found beneficial. Indeed, disappointing results have been obtained in clinical trials with anti-TNF-α therapy [anti-TNF therapy against congestive heart failure (ATTACH), randomized etanercept worldwide evaluation (RENEWAL)] and have called the beneficial effects of cytokine inhibition into question (5, 11, 18, 26).

Proinflammatory cytokines are not constitutively expressed in the normal heart (13, 14), but their expression can be triggered after myocardial infarction (4, 10) by tissue injury (19) and mechanical stress (14). Early after MI, cytokine production participates in the recruitment of inflammatory cells, contributing to the natural process of myocardial healing. Acute treatments that inhibit cytokine production after MI can affect this beneficial early response and therefore aggravate the prognosis of MI.

The role of cytokines in heart failure has been investigated either during the very early time period following infarction (first 48 h) (9, 16) or in the advanced phase of the development of congestive heart failure (1, 12, 15), but very few data are available concerning the phenomena that occur in the myocardium within the first week after MI. From the literature it appears that after an initial raise in the infarcted region of the myocardium, the cytokine level decreases to return to basal values after 24–48 h (9). Interestingly, a study by Ono et al. (22) has shown that TNF-α gene expression peaks 1 wk after...
infection in rats and decreases rapidly thereafter. Irwin et al. (10) have extended these data by showing that TNF-α mRNA and protein are persistently expressed by myocytes in the noninfarcted regions of the myocardium from 1 day to 5 wk after coronary artery ligation. Finally, we have demonstrated that the TNF-α protein is transiently overexpressed 7 days after infarction in rats and that the inhibition of this cytokine reduces cardiac alterations and improves the long-term prognosis of MI (2). Nevertheless, these observations were made on an extreme experimental model in which infarction was obtained by permanent ligation of the left coronary artery, inducing massive and transmural necrosis of the LV free wall.

To verify whether myocardial overexpression of cytokines also occurs within the first week after infarction following an episode of MI and reperfusion, we have evaluated the pattern of expression of diverse pro- and anti-inflammatory cytokines after in vivo temporary coronary occlusion in rats. Moreover, using echocardiography, we have followed, during the same period of time, the evolution of cardiac dysfunction as well as the development of cardiac remodeling.

**MATERIALS AND METHODS**

**Experimental myocardial infarction.** Adult male Wistar rats (250–350 g body wt; Charles River) were maintained on a standard diet. The care of the rats was approved by the European Communities Council Directive Animal Care and Use Committee and performed in accordance to their guiding principles (European Communities Council Directive L358/86/609/EEC). All protocols involving living animals were performed under license from the French Ministry of Agriculture (license number A 38018). Rats were anesthetized intra- periorethaneously with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg). They were rapidly intubated and mechanically ventilated (tidal volume, 1 ml/100 g body wt; ventilation rate, 65 strokes/min) by a constant-volume ventilator (Model 682, Harvard Apparatus, Edenbridge, UK) with a mixture of isoflurane (0.5%; AErrane, Lessines, Belgium) and oxygen (20%) in room air (79.5%). A left thoracotomy was performed at the fourth intercostal space, and the heart was briefly exteriorized by digital pressure on the chest wall. The left coronary artery was then ligated at 1 to 2 mm from its origin with a S-0 silk suture (Autosuture, Tyco Healthcare, Elancourt, France). The heart was quickly returned to the chest cavity. After 1 h occlusion, the ligation was removed and the left coronary artery reperfused. The chest cavity was compressed to remove any air before being hermetically sealed. Isoflurane anesthesia was then stopped and Antisedan (1.7 mg/kg im) was injected to abolish the effects of ketamine.

The overall mortality in this model was 30% for the ischemic group; about 80% of this mortality occurred during the surgical procedure, mainly because of pulmonary edema or ventricular fibrillation, and the remaining 20% occurred within the first 24 h after surgery.

Rats were randomly assigned to various groups according to the duration of left coronary reperfusion: from 1 h (day 0 + 1 h) to 10 days. Sham-operated animals were subjected to the same surgical procedure, but the ligation remained untied.

**Experimental design.** Functional, morphological, and biochemical modifications of the myocardium were evaluated 5, 7, 8, and 10 days after the initial surgical protocol.

Functional and morphological cardiac modifications were regularly monitored by echocardiography. Before euthanasia, the evaluation of cardiac function was completed in vivo, and the passive compliance of the LV was assessed thereafter ex vivo. IL-1β, IL-6, IL-4, IL-10, cytokine-induced neutrophil chemoattractant (CINC)2, CINC3, and macrophage inflammatory protein (MIP)-3α were assessed by ChemiArray on cardiac tissue samples, and TNF-α myocardial level was assayed by ELISA.

**Echocardiographic measurements.** Two-dimensional targeted M-mode recordings were obtained from the short-axis view of the heart at the level of the papillary muscle. TM-mode measurements of the LV end-systolic diameter (LVESD) and end-diastolic diameter (LVEDD) internal dimensions and of the LV anterior wall thickness (AWth) and posterior wall thickness (PWth) were made according to the American Society of Echocardiography recommendations.

The percentage of LV fractional shortening (LVFS) was calculated as a percentage = [(LVEDD − LVESD)/LVEDD] × 100. Anterior and posterior wall shortening (AWsh and PWsh, respectively) were calculated as AWsh (in %) = [(end-systolic AWth − end-diastolic AWth)/end-diastolic AWth] × 100 and PWsh (in %) = [(end-systolic PWth − end-diastolic PWth)/end-diastolic PWth] × 100.

**In vivo measurement of cardiac function.** Invasive hemodynamic studies were performed 5, 8, and 10 days after temporary coronary artery ligation, just before euthanasia. Animals were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) and ventilated via a tracheal cannula using 0.5% isoflurane as described in Experimental myocardial infarction. Body temperature was kept constant at 37°C with a heating blanket connected to a rectal thermocouple (Homeothermic Blanket System, Harvard Apparatus). A phenylephrine (PE)-10 catheter was inserted into the right femoral vein to allow volume overload with a thermostated saline solution. Mean arterial blood pressure (MAP) was measured via a PE-50 catheter (left carotid) connected to a pressure transducer (Statham P23XL, Hugo Sacks Electronik, Hugstetten, Germany), and another PE-50 catheter was inserted into the LV via the right carotid artery to monitor LV end-diastolic pressure (LVEDP), LV developed pressure (LVPD), heart rate (HR), and maximal and minimal first derivatives of developed pressure (dP/dt max and dP/dt min, respectively).

After baseline measurements had been carried out over a 10-min period to reach a steady state, thermostated (37°C) saline solution was infused into the femoral vein at a constant flow rate of 17 ml·kg−1·min−1 for 1 min (7). This volume overload was designed to reveal underlying cardiac dysfunction.

**Ex vivo determination of LV pressure-volume curves.** At the end of the invasive hemodynamic study, passive pressure-volume characteristics of the LV were evaluated postmortem, as previously described by Fletcher et al. (7). A saturated solution of potassium chloride was infused into the vena cava until the heart stopped. The heart was then excised, and a cannula, connected to a pressure transducer (Statham P23XL, Hugo Sacks Electronik) and a peristaltic pump (Minipuls 3, Gilson), was inserted 5 mm into the LV through the aorta. The right and left atrioventricular junctions, the pulmonary artery, and the vena cava were ligated, and physiological saline was infused in the LV at a constant flow rate of 0.68 ml/min while intraventricular pressure was continuously monitored from 0 to 30 mmHg. The operating LV end-diastolic volume was determined from the LV pressure-volume curve and defined as the volume on the pressure-volume curve corresponding to a filling pressure equal to in vivo end-diastolic pressure under basal conditions.

**Assessment of infarct size and cardiac geometry.** After the ex vivo evaluation of LV passive compliance, hearts were frozen in liquid nitrogen and cut at −20°C with a cryostat (Microm HM505E, Microm International, Walldorf, Germany). Three transverse sections (20 μm thick) were obtained at 5.36 mm from the base of the ventricle of each frozen heart. Each frozen section was stained using nitro blue tetrazolium chloride (0.04% in 0.05 mol/l sodium succinate buffer, pH 7.6), as described by Nachlas and Schnitka (20). Necrotic and nonnecrotic tissues were distinguished by the absence or presence of staining, respectively, and the endocardial and epicardial lengths as well as total areas of necrotic and noninfarcted muscle were estimated by planimetry (23). Briefly,
the lengths of necrotic tissue and noninfarcted muscle of the endocardial and epicardial surfaces of each histological section were numerically summed separately, as well as the endocardial and epicardial circumferences. Cardiac architecture was evaluated as described by Sulpice et al. (25). The ratio of the sums of the scar lengths and surface circumferences \( (\times 100) \) defines the infarct size.

The ratio of the LV cavity area to the LV entire area corresponds to an index of the LV cavity dilation [dilation index (DI)]. The thinning index (TI) was defined as the ratio of the thickness of the infarcted wall to the thickness of the septum. The infarct expansion thinning index (TI) was defined as the ratio of the thickness of the infarcted wall to the thickness of the septum. The infarct expansion index (EI) was estimated from the ratio of DI to TI.

**Cytokine array and estimation of myocardial TNF-\( \alpha \) by ELISA.** After the collection of histological sections, frozen heart samples \((200 – 400 \text{ mg})\) were crushed in liquid nitrogen and homogenized in Tris (5 mM)-EDTA (2 mM) buffer (pH 7.4) containing a protease inhibitor cocktail 1/200 (P2714, Sigma, L’Isle d’Abeau Chesnes, France) and Triton X-100 (0.5%). After incubation for 2 h at 4°C, this inhibitor cocktail 1/200 (P2714, Sigma, L’Isle d’Abeau Chesnes, France) and Triton X-100 (0.5%). After incubation for 2 h at 4°C, this suspension was centrifuged for 30 min at 14,000 g at 4°C to remove cellular debris. Protein content was measured using a BCA protein assay kit (Pierce). TNF-\( \alpha \) (in pg/mg protein) was detected using a sandwich ELISA kit (rat TNF-\( \alpha \)/TNFSF1A, Duoset, R&D Systems, Abingdon, UK).

IL-1\( \beta \), IL-6, IL-4, IL-10, CINC2 and CINC3, and MIP-3\( \alpha \) were semiquantitatively assessed using the ChemiArray Rat Cytokine Antibody Array Kit (Chemicon-Millipore). For each group, the same amount of cardiac extract from three different hearts was mixed and incubated with the membrane. Membranes were scanned and blot intensities (in arbitrary units) were measured using ImageJ 1.37. Each blot intensity was expressed as a percentage of a specific positive internal standard. Expression modifications are considered significant when the expression level is at least 50% different from baseline level.

**Statistical analysis.** Values are expressed as means \( \pm \) SE. One-way ANOVA was performed to determine significant differences between groups. The significance of the difference between the means of the groups was tested with Fisher a posteriori-protected paired least significance test. \( P = 0.05 \) was considered the threshold of statistical significance.

### RESULTS

**Infarct size and cardiac geometry.** Infarct size was similar among rats that underwent 5–10 days of reperfusion (Table 1). In MI groups, the TI was reduced by 50% within the first 5 days after surgery and remained constant thereafter (Fig. 1A). The DI progressively increased from day 8 after infarction, and this effect was significant at day 10 (Fig. 1B). The EI was significantly increased from day 5 after surgery and reached a maximum at day 9 (Fig. 1C).

**Echocardiographic measurements.** LVFS (Fig. 2A) as well as AWsh and PWsh (Fig. 2B) was significantly decreased within the first 5 days after reperfused MI and remained constant up to day 10. LVEDD (Fig. 2C) increased progressively during the first 10 days after reperfused MI.

**Hemodynamic measurements.** MAP was significantly decreased after volume overload at day 8 compared with controls. LVEDP was significantly increased from day 8 under basal conditions as well as after volume overload. LVDP was significantly decreased under basal conditions as well as after volume overload from day 5 after surgery. \( \frac{\text{dP}}{\text{dt}} \) was significantly increased from day 8 after reperfusion. \( \frac{\text{dP}}{\text{dt}} \) was significantly decreased after volume overload from day 8. \( \frac{\text{dP}}{\text{dt}} \) was significantly decreased under basal conditions at day 10 and after volume overload from day 5. HR was significantly decreased at day 10 under basal conditions as well as after volume overload.

**Passive compliance.** A significant rightward shift of MI curves compared with controls was observed from day 5 after ischemia-reperfusion (Fig. 3). Operating LV end-diastolic volume was also significantly increased in the ischemic hearts from day 5 after surgery (Table 1).

**Myocardial level of TNF-\( \alpha \).** Myocardial TNF-\( \alpha \) measured by ELISA was significantly increased 1 h after MI compared with nonoperated animals and returned to baseline after day 1. A significant and transient increase in the TNF-\( \alpha \) myocardial level was observed 8 days after reperfused MI (Fig. 4).

### Table 1. Infarct size and hemodynamic variables at baseline and after 1 min infusion of saline solution

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Day 5</th>
<th>Day 8</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infarct size, %</td>
<td></td>
<td>38.1±8.4</td>
<td>40.3±3.6</td>
<td>38.9±5.6</td>
</tr>
<tr>
<td>OLVEDV, ml</td>
<td>0.03±0.02</td>
<td>0.18±0.05*</td>
<td>0.23±0.03*</td>
<td>0.24±0.04*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>84.38±0.04</td>
<td>74.73±5.49</td>
<td>75.32±3.67</td>
<td>82.13±4.57</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>87.87±5.68</td>
<td>75.04±4.11</td>
<td>73.41±3.71*</td>
<td>79.25±5.27</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>1.32±2.12</td>
<td>4.38±2.05</td>
<td>7.01±2.05*</td>
</tr>
<tr>
<td>Volume overload</td>
<td>5.71±2.06</td>
<td>12.16±2.98</td>
<td>17.30±2.48*</td>
<td>20.83±3.77*</td>
</tr>
<tr>
<td>Baseline</td>
<td>104.28±1.69</td>
<td>83.27±6.04*</td>
<td>83.51±4.75*</td>
<td>81.17±6.89*</td>
</tr>
<tr>
<td>Volume overload</td>
<td>95.90±5.69</td>
<td>71.45±4.69*</td>
<td>67.33±6.37*</td>
<td>63.46±8.61*</td>
</tr>
<tr>
<td>( \frac{\text{dP}}{\text{dt}}_{\text{max}}, ) mmHg/s</td>
<td></td>
<td>2,124.50±163.35</td>
<td>1,714.84±133.37</td>
<td>1,730.55±92.64</td>
</tr>
<tr>
<td>Baseline</td>
<td>1,882.67±193.05</td>
<td>1,376.43±107.57*</td>
<td>1,274.36±76.12*</td>
<td>1,182.57±265.17*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>2,317.83±87.28</td>
<td>1,802.51±146.77</td>
<td>1,809.41±103.61</td>
<td>1,781.40±154.18</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SE. \( n = 6–11 \) rats per group. MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; \( \frac{\text{dP}}{\text{dt}}_{\text{max}} \) and \( \frac{\text{dP}}{\text{dt}}_{\text{min}} \), maximal and minimal first derivatives of developed pressure; OLVEDV, operating left ventricular end-diastolic volume. \( *P < 0.05 \) vs. control.
Cytokine array. Myocardial levels of IL-1β, IL-6, MIP-3α (Table 2), CINC2 (Fig. 5A), and CINC3 (Fig. 5B) were significantly increased after MI and reached a maximum at day 8. Myocardial expression of the anti-inflammatory cytokines IL-4 and IL-10 was not significantly affected by MI (Table 2).

DISCUSSION

Our model of experimental reperfused myocardial infarction induces, from day 5 after surgery, profound modifications of myocardial structure including expansion and thinning of the infarcted zone and dilation of the LV cavity. Moreover, a major contractile dysfunction, characterized by a 60% reduction of the echocardiographic index of ejection fraction (i.e., LVFS), is also detected from day 5 of postinfarction. Finally, our results indicate that a burst of proinflammatory cytokine expression (TNF-α, IL-1β, IL-6, CINC2, CINC3, and MIP-3α) develops in the myocardium 8 days after temporary artery ligation. Moreover, this peak of cytokine expression occurs simultaneously with the paroxysm of early cardiac dysfunction and ventricular compliance alteration, suggesting a relation of cause and effect between these phenomena.

It is now well established that MI leads to, within a few hours, the development of an inflammatory process that is exacerbated when the ischemic tissue is reperfused. This initial inflammatory process, which is limited to the first 24–48 h after infarction, is beneficial to the heart (24) although it induces a transient overproduction of diverse cytotoxic agents. Several studies have indeed demonstrated that this early inflammatory process contributes to myocardial healing and enables the restoration of some structural cohesion in this fragile territory (6). At this stage, activation of chemokine and cytokine cascades results in recruitment of leukocytes into the...
infarcted region of the heart. Neutrophils and macrophages clear the wound of dead cells and matrix debris. Activated macrophages release cytokines and growth factors, leading to the formation of granulation tissue (8).

Our results confirm the existence of this initial inflammatory process in our model since myocardial TNF-α was shown to be overexpressed 1 h after ischemia-reperfusion and returned to baseline within the first 24 h (Fig. 4).

The second phase of infarct healing, which partly overlaps the first one, is named the proliferative phase and is characterized by the production of extracellular matrix proteins by activated myofibroblasts and the formation of an extensive microvascular network. The maturation of the scar follows (7–14 days) as fibroblasts undergo apoptosis and a collagen-based scar is formed.

In a previous study (2) we have seen a secondary peak of myocardial TNF-α occurring 7 days after permanent regional ischemia in rats. Moreover, we have demonstrated that this secondary transitory expression of TNF-α occurred simultaneously with cardiac dysfunction and contributed to the subsequent development of chronic heart failure. The results presented here indicate that a similar phenomenon occurs in our model of ischemia-reperfusion. Indeed, 8 days after the surgical procedure, i.e., at the beginning of the maturation phase, TNF-α accumulates transiently in the myocardium. Moreover, our study shows that other proinflammatory cytokines follow the same expression profile, whereas the myocardial level of IL-4 and IL-10, two anti-inflammatory cytokines, remained unchanged. Finally, our 10-day follow-up of cardiac function and geometry reveals that cardiac mechanical dysfunction, infarct expansion (EI), and the alteration of LV passive compliance present a maximum during this particular time frame.

These results suggest that the delayed peak of proinflammatory cytokines after reperfused myocardial infarction (8 days postligation in our rat model) might be one of the possible triggers of postinfarct cardiac dysfunction and could therefore contribute to the evolution toward heart failure. Nevertheless, the main limitation to the present study is the lack of direct evidence of a relation of cause and effect between these two

![Fig. 3. LV pressure-volume curves 5, 8, and 10 days following transient ischemia. LV pressure-volume curves were determined on ex vivo-arrested perfused hearts. Values are means ± SE; n = 9–16 rats per group. †P < 0.001 vs. myocardial ischemia (MI) day 5 (d5), MI day 8 (d8), and MI day 10 (d10).](#)

### Table 2. Cytokine array: ChemiArray

<table>
<thead>
<tr>
<th></th>
<th>Nonoperated</th>
<th>MI Day 3</th>
<th>MI Day 5</th>
<th>MI Day 8</th>
<th>MI Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>2.80</td>
<td>3.64</td>
<td>3.80</td>
<td>5.60*</td>
<td>6.07*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.70</td>
<td>5.62*</td>
<td>4.02*</td>
<td>7.16†</td>
<td>6.26*</td>
</tr>
<tr>
<td>IL-4</td>
<td>3.97</td>
<td>6.33</td>
<td>2.11</td>
<td>4.98</td>
<td>3.33</td>
</tr>
<tr>
<td>IL-10</td>
<td>6.31</td>
<td>7.08</td>
<td>8.29</td>
<td>8.84</td>
<td>9.24</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>5.23</td>
<td>7.18</td>
<td>5.55</td>
<td>8.97*</td>
<td>7.36</td>
</tr>
</tbody>
</table>

Cytokine levels are expressed as percentages of blot intensity compared with positive controls of the membrane. MI, myocardial ischemia; MIP, macrophage inflammatory protein. *50% increase compared with nonoperated. †400% increase compared with nonoperated.

![Fig. 4. Evolution of myocardial TNF-α during the first 10 days following transient ischemia. ELISA assay on 3–9 hearts/group. Values are means ± SE. **P < 0.01 vs. sham-operated rats.](#)

![Fig. 5. Pattern of evolution of cytokine-induced neutrophil chemoattractant (CINC) during the first 10 days following transient ischemia. CINC2 (A) and CINC3 (B) are shown. Blot intensities are expressed as a percentage of positive-specific internal standards.](#)
phenomena. Therefore, further studies using specific inhibitors of diverse proinflammatory cytokines are needed to confirm this proposal.

**GRANTS**

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